Transfusion Medicine and Hemotherapy

Review Article

Transfus Med Hemother 2024;51:430–438
DOI: 10.1159/000538972

Received: June 5, 2023
Accepted: April 16, 2024
Published online: June 5, 2024

# Advancing Transfusion Medicine through Raman Tweezers Spectroscopy: A Review of Recent Progress and Future Perspectives

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**Keywords**

Raman spectroscopy · Optical tweezers · Red blood cells · Transfusion · Intravenous fluids

**Abstract**

***Background:*** Raman tweezers spectroscopy (RTS) is a powerful tool that combines optical tweezers and Raman spectroscopy to study single living cells. RTS has become increasingly popular in biomedical and clinical research due to its high molecular specificity and sensitivity, which enable the study of cell viability, cell deformation, cell-protein, cell-nanoparticle, cell-cell interaction, etc. In transfusion medicine, RTS can give valuable insights into the storage lesions and effects of various preservatives and intravenous fluids on blood cells. ***Summary:*** By analyzing the Raman spectra of individual blood cells, RTS can detect changes in the cellular blood components which can be used to monitor the quality of blood products during storage and transfusion. The present review article highlights the principle and clinical applications of RTS in transfusion medicine. ***Key Messages:*** Raman spectroscopy is a versatile analytical method for biomedical research. Combining the Raman spectroscopy method with the optical tweezers technique will allow us to explore the dynamics of live single cells in their physiological medium.



## Introduction

Blood is the prerequisite of life, and it is continuously circulating through the blood vessels by delivering nutrition and oxygen to the body and removing waste materials. There are numerous research projects underway in the biomedical realm. Several research studies have explored the application of principles of physics in blood storage and preservation to improve the quality and safety of stored blood. For the study of blood components, Raman spectroscopy might be a precise method.

The molecular specificity and the higher sensitivity of Raman spectroscopy have been widely accepted as an important characterization tool for biomedical and clinical applications [1–3]. The details on the chemical compositions, molecular structure, and the interaction of molecules in the cells and the tissues can be provided by this technique [4, 5]. The change in molecular composition in blood components may affect the quality, and this will be evident in their Raman spectra. The changes in spectra are unique to the specific intracellular modifications, and they are considered Raman markers. The Raman spectroscopy method relies on the inelastic scattering of light by the substance. The existence of inelastic scattering of light was invented by Sir C.V. Raman and K.S. Krishnan. Sir C.V. Raman won the Nobel Prize in 1930 for the invention of the inelastic scattering of light, which is known as the Raman effect [6]. Raman fingerprint of cells can provide knowledge of the cell's

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structure, integrity as well as biochemical changes [7]. The Brownian movement of a single living cell suspended in a physiological media severely impedes direct probing. In 1970, Ashkin [8] introduced a new technique called optical tweezers for the study of microscopic and submicroscopic particles like droplets and cells. The same technique was introduced in the biological field for the study of single-live blood components, bacteria, DNA, etc. The optical tweezers use an extremely focused laser beam to trap moving cells at the laser point [8, 9]. This technique was widely used for the membrane-related studies of different cells. Wagner and colleagues [10] employed holographic optical tweezers to investigate the possible adhesion between red blood cells (RBCs). Through experiments, they have demonstrated the role of RBCs in the blood coagulation process. Elevating the internal $Ca^{2+}$ concentration results in RBCs adhering to each other, much like the adhesion induced by lysophosphatidic acid stimulation. Likewise, optical tweezers were employed to investigate variations in the RBC aggregation characteristics among different RBC age groups through single-cell experiments conducted in vitro [11]. Many research groups explored the elastic characteristics of various cells, primarily RBCs [12–14]. Optical tweezers were used for the examination of malaria-infected RBCs, neuroacanthocytosis syndromes, etc. [15, 16]. Raman tweezers spectroscopy (RTS) is the result of the fusion of optical tweezers and Raman spectroscopy, and this is a micro-Raman spectroscopy technique used to analyze single living cells and other particles suspended in an aqueous/physiological environment [17]. The main applications of RTS in the medical field include the studies of cancer cell lines, erythrocytes, leukocytes, and thrombocytes at a single-cell level [18]. RTS was also employed in the research of ABO blood typing [19]. In the case of Raman tweezers system, a laser beam is utilized to arrest the micron-sized particles (e.g., a living cell), while the same or another laser simultaneously excites the trapped sample and generates inelastically scattered radiation which is detected and recorded using a spectrometer resulting into Raman spectrum [20].

This integrated system offers a solution to avoid chemically altering cells and instead provides insights into their natural physiological state [21]. Consequently, it simplifies the manipulation of cells for necessary research. Another advantage of RTS technology is its minimal sample requirement for the Raman spectra measurement. Typically, biological research and clinical tests involve the study of groups of cells. However, single-cell studies can yield more significant and reliable results than examining cell clusters. RTS requires a tightly focused laser beam to immobilize living cells in a specific location within a sample holder containing a physiological medium for spectra recording. Focusing of light is necessary in order to achieve the required trapping force for the optical tweezer's functionality. If the light is not focused into a smaller spot, the stability of the trap will be reduced and there will be an increased likelihood of the cell escaping from the optical trap. Determining the appropriate laser wavelength for trapping and spectral recording poses a major challenge in single-cell research, as the laser beam can damage the cells [22, 23]. Various research groups have conducted experiments to identify the optimal laser wavelength for both trapping and probing purposes [24].

In the medical field, intravenous (IV) fluid infusion is a life-saving intervention. The need for an IV fluid transfusion may be urgent in a variety of circumstances. Different kinds of IV fluids are used for transfusions [25]. Colloid solutions and crystalloid solutions are the two categories for IV fluids, and research has been conducted to determine which category is best for IV transfusion [25]. Many groups of researchers have carried out studies on how IV fluids affect blood cells [26, 27]. The biochemical alterations that IV fluids have on cells can be investigated using the RTS technique. The current review paper includes a detailed description of the RTS technique and its application for the study of various types of blood cells that appeared in the literature during the past 20 years.

## The Basic Principle of RTS

The instrument used for the RTS is a combination of two very popular analytical methods, Raman spectroscopy and optical tweezers for the study of dielectric particles in their respective medium. Raman spectroscopy investigates inelastically scattered light from the target samples, which can provide enough details about the molecular makeup, particularly the functional groups found in biological and chemical analytes [28, 29]. In Raman spectroscopy, a single wavelength light is allowed to impinge on the analytes, and the resulting inelastically scattered light is detected and recorded by the Raman spectrometer. Elastic and inelastic scattering are both a part of light scattering. While most of the light that is scattered is elastic and has the same frequency as the light that was incident, a tiny amount of light with lower or increased wavelength than the incident light is inelastically scattered. The vibrational energy-level diagram shown in Figure 1a illustrates all the above scattering processes. In the first case, the excited molecule can relax to the ground level by releasing its energy in the form of a light photon (quantized light) with an equal wavelength as the incident light (green color arrow), and this elastic scattering process is known as Rayleigh scattering. The molecule can relax to a vibrational state and emit a photon that has a higher wavelength than the incident photon. This is called Stokes-Raman scattering (red color

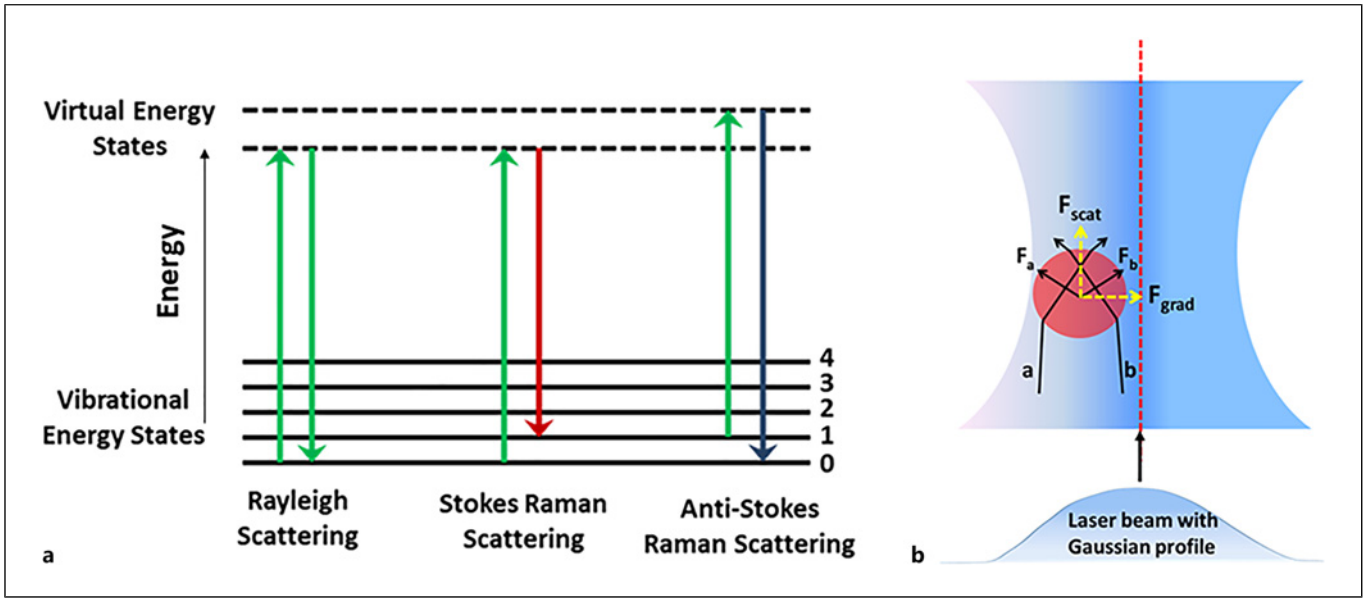


**Fig. 1. a** An illustration of the Rayleigh and Raman scattering processes (green color arrow pointing up indicates incident light, green color arrow pointing down indicates Rayleigh scattering, red color arrow indicates Stoke's Raman scattering, and blue color arrow indicates anti-Stoke's Raman scattering). **b** Ray diagram representation of optical trapping of a single dielectric sphere in a tightly focused laser beam. a and b represent two rays and $F_a$, $F_b$ force exerted by a and b; $F_{scat}$ (scattering force), $F_{grad}$ (gradient force).

arrow). The molecule in the last scenario is the one that has already undergone vibrational excitation and is now in its lowest possible state, where it emits a photon with less wavelength than the incident photon. The resulting scattering is known to be anti-Stokes-Raman scattering (blue color arrow) [29]. At room temperature, the majority of the molecules will be in the ground state, and then the probability of anti-Stokes-Raman scattering is very less. Therefore, the majority of Raman experiments solely take into account Stoke's scattered photons [30]. The *x*-axis of Raman spectra is conventionally represented in wavenumber or Raman shift ($cm^{-1}$). The formula for converting wavenumber ($cm^{-1}$) to wavelength (nm) is provided in the online supplementary material (for all online suppl. material, see https://doi.org/10.1159/000538972).

It is necessary to halt or trap micron-sized particles or cells in the medium for further examination to prevent their natural Brownian mobility. Using an extremely focused laser beam, optical tweezers can manipulate dielectric particles [31–33]. The scattering and gradient forces play crucial roles in optical trapping. The gradient force caused by the tightly focused laser beam works as an attracting force and pulls the sphere toward the higher intensity area, while the scattering force pushes the sphere in the direction of propagation. Therefore, since the light gradient heads toward the center, a focused laser can set a trap [34]. A tightly focused laser beam by a high numerical aperture (N.A. 1.3) microscope objective will make the intensity around the laser focus steep; in this case, the gradient force dominates the scattering force, and this will result in a more stable optical trap [35, 36]. Figure 1b shows the schematic diagram of an optically trapped dielectric particle.

RTS technique is a well-established technique for single-cell analysis [37, 38]. In the experimental part, the Rayleigh (elastic) scattered light has been rejected using an edge filter and recorded only the Raman (inelastic) scattered light. The resultant Raman spectrum can be used as the "fingerprint" of those analytes to identify it. Raman spectroscopy is a versatile analytical technique accepted by all science disciplines since it can distinguish between molecules that are quite similar to one another. This is because the Raman spectrum contains unique Raman signatures (fingerprints) for each analyte. The schematic of the dual beam Raman tweezers setup is given in online supplementary Figure S3. The 1,064 nm laser was used for trapping the cell and the 785 nm laser for probing. In the case of a single beam setup, the only difference is the absence of a 1,064 nm laser; here, 785 nm will perform both trapping and probing [39].

Infrared spectroscopy is another analytical method that leverages the benefits of molecular vibrational transitions. It is a widely employed approach for deducing the composition and identifying the structure of chemical compounds [40]. Application of IR spectroscopy in biological research is very uncommon due to the presence of water in biological samples. IR measurements are usually done for dried materials (free from water) [41]. The presence of water will interfere with the IR spectrum

of water-containing samples like tissues, cells, etc. Hence, Raman spectroscopy techniques are highly suitable for biomedical research.

## RTS in Transfusion Medicine

This review paper consists of the Raman tweezers study of different live single healthy human cells under different stressful conditions, which includes different laser wavelengths, chemicals, nanoparticles, IV fluids, etc. In the case of biological samples especially in single-cell studies, the selection of proper laser sources and the laser powers are crucial. Therefore, many research teams carried out the laser power and wavelength-dependent Raman tweezers studies for biological samples [23, 42, 43]. The detailed description about the impact of lasers on single living cell was given in the online supplementary material. In this section, the Raman spectra of optically trapped RBC, platelet, and WBC are provided in online supplementary Figure S1. In addition, the ability of Raman tweezers technique to probe oxygenation as well as deoxygenation of hemoglobin in RBC is explained by citing the changes happening for the oxygenation markers in the RBCs suspended in normal saline. As shown in online supplementary Figure S2, Raman markers at 1,209 $cm^{-1}$ and 1,222 $cm^{-1}$ (the wavenumber [$cm^{-1}$] to wavelength [nm] conversion formula is provided in the online suppl. material) can give an overview of the oxyhemoglobin variations happening inside RBC under the influence of any exogenous media/agents. The wavenumber to wavelength conversion is also provided in Table 1 given in the online supplementary material.

## The Effect of Different External Chemical Agents on Single-Living Blood Cells

Several research groups have investigated the processes of RBC deoxygenation, oxidative stress, and hemoglobin denaturation upon the influence of exogenous chemicals like alcohol, nanoparticles, bisphenol-A (BPA), etc. In clinical settings, alcohol is routinely used as a sterilizing agent to clean wounds, where alcohol helps disinfect the affected region. In the field of transfusion medicine, we defer the donors who have consumed alcohol, so the impact of alcohol on RBCs is an interesting research question. The interaction of RBCs and alcohol by using the Raman tweezers technique was first investigated by Deng et al. [44]. The RBCs were treated with different percentages of alcohol for checking the differences in Raman bands. RBC treated with alcohol has a remarkable intensity decrease of the peak positioned at the 752 $cm^{-1}$ region [44, 45]. Lukose et al. [46] conducted a follow-up study on the effects of alcohol on RBCs, and they provided some new details on the oxy-deoxy condition of RBCs. In addition to deoxygenation, hemoglobin denaturation and depletion were observed when the RBCs were treated with a higher concentration of ethanol, which is clear from the reduced intensity of porphyrin breathing mode (752 $cm^{-1}$). The ethanol-treated RBCs have remarkable intensity variations in C-H deformation regions and spin marker regions (1,209 $cm^{-1}$, 1,222 $cm^{-1}$, 1,544 $cm^{-1}$, 1,561 $cm^{-1}$, and 1,636 $cm^{-1}$) [46]. The same research group has studied the influence of BPA on single RBCs with the help of the RTS technique.

BPA is an intermediate that is used in combination with other chemicals to manufacture plastics, and it is used in many medical devices and blood tubing. It is an endocrine disrupter, and it will promote carcinogenic activities and other major health disorders. The researchers have conducted the study based on the consequence of BPA which is extracted from the thermal paper on the live RBCs. They have concluded that the BPA-treated RBCs have reduced Raman band intensity of porphyrin breathing mode (752 $cm^{-1}$) and phenylalanine (999 $cm^{-1}$); also, the oxy-deoxy marker bands have a noticeable intensity variation [47].

Bankapur et al. [48] investigated the effect of silver nanoparticle (AgNP)-induced stress on individual human mesenchymal stem cells. The results obtained from the single-cell experiment show that the AgNPs induce oxidative stress on the mesenchymal cells and will lead to cell membrane and DNA damage, and a higher concentration of AgNPs can lead to cell death [48]. Similarly, the silver and gold nanoparticle-treated single RBCs also showed oxidative stress, leading to cell damage [49]. Zachariah et al. [39] experimented on single-live RBCs to understand the oxidative stress caused by OH radicals. Raman bands at 499 $cm^{-1}$, 520 $cm^{-1}$, and 543 $cm^{-1}$ (S=S stretching mode) and 640 $cm^{-1}$ and 665 $cm^{-1}$ (C-S stretching mode) have shown increased intensity due to oxidative stress [39]. The cell damage of single RBCs was studied by treating the cells with different external agents like free radicals, glucose, AgNPs, heat, and osmotic shock [50]. The Raman bands showed the conversion of the hemoglobin from the oxygenation state to the deoxygenation state, otherwise known as R to the T state [50]. The impact of RBCs in an acute hypoxic condition was studied by Chowdhury and Dasgupta [51]. They artificially created the acute hypoxic condition by purging nitrogen over a while from 15 to 60 min; after that, the sample was equilibrated with the atmospheric condition for 10 min. This oxygen-deficient condition was almost similar to the atmospheric condition at high altitudes, so the study can be correlated with the high altitude syndrome and pulmonary diseases. The Raman spectra of exposed and unexposed RBCs were showing changes in oxygenation

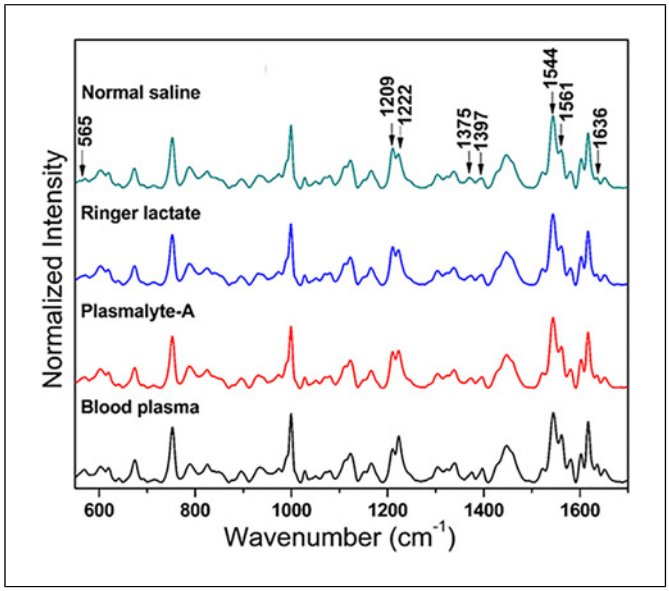


**Fig. 2.** Raman spectra of living RBCs in various IV fluids.

marker bands (569 $cm^{-1}$, 1,220 $cm^{-1}$, 1,636 $cm^{-1}$) and deoxygenation marker bands (1,210 $cm^{-1}$, 1,547 $cm^{-1}$) [51].

IV fluid therapy is common in hospitals. Different forms of IV fluids, such as NS3%, NS0.45%, dextrose 5%, DNS, hydroxyethyl starch (HES), Ringer lactate, Plasmalyte-A, etc., are routinely in use for fluid resuscitation and during therapeutic apheresis by the transfusion medicine specialists. Blood cells interact differently depending on tonicity, pH, and minerals contained in IV fluids. Therefore, it is crucial to study how blood cells interact with these fluids. Lukose et al. [52] did comparison research to examine the effect of normal saline (0.9%) on individual RBCs concerning human blood plasma. The remarkable spectral variations were observed in the Raman bands at 1,209 $cm^{-1}$, 1,222 $cm^{-1}$, 1,544 $cm^{-1}$, and 1,561 $cm^{-1}$. Normal saline-treated RBCs have a change of oxygenated hemoglobin (R state) to deoxygenated state (T state) [52]. The same research team extended their work by examining the impact of various IV fluids on RBCs. They conducted these experiments by using both crystalloid and colloidal solutions. Figure 2 shows the Raman spectra of RBCs treated with various IV crystalloid fluids and blood plasma [53]. The major spectral variations were observed in the oxy-deoxy marker bands, and the cells were deoxygenated in crystalloid solutions. The $FeO_2$ stretching band at 564 $cm^{-1}$ has a reduced intensity in normal saline and Ringer lactate.

Lukose et al. [54] have studied how the Raman spectra of RBCs changed under various tonicity conditions. Hypertonic saline (3%), hypotonic saline (0.45%), and blood plasma were used to treat the RBCs. The blood plasma serves as the study's control. In a hypertonic environment, heme aggregation leading to protein denaturation was seen. In this condition, the RBCs shrank and underwent echinocyte conversion. When using hypotonic saline, discoid RBCs transformed into spherocytes. The deoxygenation of the RBCs was evident in both hypertonic as well as hypotonic solutions. The conversion of oxyhemoglobin to deoxyhemoglobin was observed in the RBCs treated with dextrose-containing IV fluids [55]. The heme aggregation was also evident in the Raman spectra of dextrose-treated RBCs. The behavior of RBCs in HES was studied by Mithun et al. [56]. HES is a colloidal solution used for IV transfusion. The Raman spectroscopy study shows that the HES reduces the oxyhemoglobin level and induces membrane damage in the cells.

The selection of the ideal IV fluid for transfusion requires the completion of comprehensive studies. When cells were treated with various IV fluids, the Raman spectroscopy tests revealed some negative effects on the cells. Some of the IV fluids were harming the RBCs' membranes and denaturing their hemoglobin, which is a serious condition.

## Raman Spectroscopy Study of Hemoglobinopathies and ABO Blood Typing

Beta-thalassemia is a blood disorder that results from a decrease or absence in the production of beta-globin chains in hemoglobin. Early and accurate diagnosis is crucial for mitigating risks and initiating appropriate medical treatment [57]. The beta-thalassemia can be classified as beta-thalassemia major (it can cause major health problems and early death), beta-thalassemia intermedia (it will cause delayed growth, weak bones, etc.), and beta-thalassemia minor (it should not cause major health issues) [58]. So, the early diagnosis is very much important for starting the proper medication against this disorder. In 2008, De Luca et al. [59] conducted a study that centered on the spectroscopic and mechanical analysis of individual erythrocytes (RBCs) from both normal individuals and those affected by beta-thalassemia. This analysis was conducted using RTS. The live erythrocytes were trapped by a 1,064 nm Nd:YAG laser, and the spectra were recorded by 532 nm Nd-YVO laser. The Raman peak intensities representing the oxygenated Hb are very much reduced for the β-thalassemic cells. Comparing to normal erythrocytes, the beta-thalassemia-affected cells have higher photodamage.

In that research, they subjected healthy and β-thalassemic RBCs to a 15-mW laser beam for durations ranging from 3 to 150 s. Their findings indicated that when exposed for 80 s, there was a 50% reduction in the Raman signal for normal cells and an 80% reduction

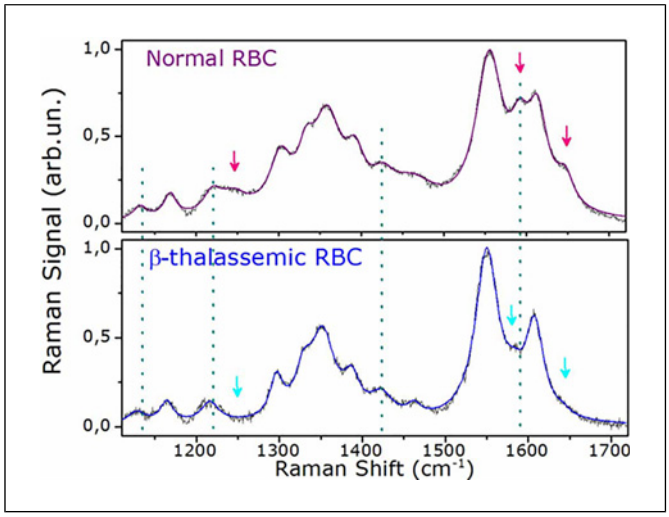


**Fig. 3.** Raman spectra of normal RBC and beta-thalassemic RBC.

for β-thalassemic cells. This suggests that β-thalassemic RBCs exhibit a higher sensitivity to photo-oxidation. In this same work, De Luca et al. [59] conducted a mechanical stretching study on the RBCs using the same setup and found that the rigidity of the cell was more in β-thalassemic cells. Figure 3 shows the Raman spectra of normal RBC and the beta-thalassemic RBC. Another RTS study was conducted by the same group, focusing on normal and thalassemic single-live erythrocytes. They have observed spectral variations in the spin marker region, C-H methine deformation region, asymmetric pyrrole half-ring stretch region, C-C symmetric stretching, and pyrrole breathing [60]. Using the Raman spectroscopy technique, Jia et al. [61] investigated the differences between beta-thalassemia major, beta-thalassemia minor, and normal RBCs. Comparing to the Raman spectra of normal RBCs, the beta-thalassemia major cells were showing reduced Raman band intensities, especially at 1,546 $cm^{-1}$, 1,603 $cm^{-1}$, and 1,619 $cm^{-1}$. The beta-thalassemia minor could not be distinguished effectively from normal cells by the research team. The normal RBCs, sickle cell disease-infected RBCs, and cord blood RBCs can be differentiated by comparing the intensities of 1,200–1,250 $cm^{-1}$ and 1,500–1,650 $cm^{-1}$ regions which are considered as the oxygenated and deoxygenated Raman marker peaks of the cells. The deoxygenation rate was comparatively more in sickle RBCs, while the experiment was carried out by changing the trapping laser powers. By increasing laser power, the deoxygenation rate of sickle RBCs was faster than normal RBCs, and at the same time, it was less in cord blood RBCs [62].

Lin et al. [19] conducted an experiment using RTS to analyze ABO blood types. Their results showed a remarkable 100% accuracy in classifying the AB blood type from other blood groups. The key distinguishing factor was the intensity of the Raman band at 753 $cm^{-1}$ (C-N-C breathing stretch vibrations in the porphyrin ring), which was significantly higher in the AB blood group compared to all the other blood groups. Single-living cell studies on ABO blood grouping were very less, and some research groups conducted surface-enhanced Raman spectroscopy techniques for the ABO blood typing studies. Wang et al. [63] made a membrane electrophoresis-based SERS substrate for the classification of different blood groups. With this technique, they have classified type A, type B, and type O blood samples. In the Raman spectra of individuals with blood type O, the most prominent intensity is observed in the spectroscopic peaks associated with tyrosine at wavenumbers 637, 644, and 1,605 $cm^{-1}$, phenylalanine at 1,001 and 1,605 $cm^{-1}$, and amide I at the range of 1,640–1,680 $cm^{-1}$. Conversely, these peaks are notably weaker in individuals with blood type B. On the other hand, the spectroscopic peaks at 882 and 1,342 $cm^{-1}$, which correspond to tryptophan, exhibit the highest intensity in individuals with blood type B, while being the weakest in individuals with blood type O.

## Raman Spectroscopy Study of Storage Lesions of Blood Components

Blood transfusion is a critical medical procedure that can save lives. It is very essential for patients suffering from conditions such as anemia, complications during pregnancy and childbirth, severe trauma resulting from accidents, or individuals undergoing surgical procedures, and requires immediate infusion of blood. Numerous research studies have been conducted to investigate the potential harm caused by storage of blood components over time. Gautam et al. [64] conducted an experiment utilizing Raman spectroscopy to investigate the degradation of RBCs over time. Throughout the storage period, an increase in both oxyhemoglobin and methemoglobin levels, as well as a decrease in membrane fluidity was observed. Additionally, a rise in the intensity of Raman bands at 569 $cm^{-1}$, 1,225 $cm^{-1}$, 1,565 $cm^{-1}$, and 1,638 $cm^{-1}$, which are associated with oxyhemoglobin, is also found, as the storage period progressed. Vardaki et al. [65] employed spatially offset Raman spectroscopy configuration to examine the changes that occur in stored packed RBCs within the storage bag. One notable advantage of their study is that it eliminates the need to extract a sample from the blood bag. The primary focus of the study was on the deoxyhemoglobin/oxyhemoglobin ratio, which is represented by the Raman bands at 1,208 $cm^{-1}$ and 1,224 $cm^{-1}$. As the storage duration increased, this ratio showed a decreasing trend. The RBC storage effect investigation was carried out by Atkins et al. [66] using Raman spectroscopy, wherein an increasing trend of oxygenation was observed in the stored RBCs.

## Conclusion

The RTS is a versatile technique for the investigation of living cells. This review paper highlights how the RTS can be used to study single-living human cells and its applications in the field of transfusion medicine. The main benefits of the Raman tweezers technique include the fact that the sample size required for the research was in the range of microliters, the sample preparation procedure was simple, and there was no sample damage both during and after the inquiry. Usually, imaging techniques are used for the studies of single cells, but the staining agents and fixatives used for the studies will adversely affect the obtained results and also will restrict the living cell studies. This technique is very useful in assessing the storage lesions of blood components.

This review paper also examined the evaluation of the interaction between RBCs and various external substances, such as BPA, nanoparticles, IV fluids, etc., employing RTS technology. In the medical field, the commonly used IV fluid was normal saline. The Raman spectra of single RBCs showed that the cells are getting deoxygenated by the interaction with normal saline. The Raman bands corresponding to the oxygenation marker were showing reduced intensity in normal saline. Similarly, the nanoparticle interaction on RBC was also causing deoxygenation of the cells. The interaction of different wavelength lasers on single living cells was also discussed in this review article. An appropriate laser wavelength for single-living cell trapping and probing was optimized from the near-infrared region. Continuous laser irradiation to a single cell will harm the cell, leading to membrane depletion, protein denaturation, and oxygen deprivation, among other effects.

The cost and the large size of the system are the main drawbacks of the RTS technique. The research articles discussed in this review article have proved that the RTS technique is highly sensitive, reliable, and one of the promising techniques for the studies of single living cells.


## Acknowledgment

Mr. Mithun Nelliat is grateful to Manipal Academy of Higher Education, Manipal, for the Dr. TMA Pai Doctoral Fellowship.


## Conflict of Interest Statement

The authors have no conflicts of interest to declare.


## Funding Sources

The authors are thankful to the Department of Biotechnology, Government of India, and Manipal Academy of Higher Education for the financial support to establish the Raman tweezers setup. Thanks are due to the DST-FIST Government of India and VGST Government of Karnataka for financial support to set up other research facilities.


## Author Contributions

Mithun Nelliat: writing original draft. Ganesh Mohan: review and editing. Jijo Lukose: writing, review, and editing. Shamee Shastry: review, editing, and supervision. Santhosh Chidangil: conceptualization, supervision, review, and editing.

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